

# Interaction of heparin with annexin V

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**Abstract** The energetics and kinetics of the interaction of heparin with the  $\text{Ca}^{+2}$  and phospholipid binding protein annexin V, was examined and the minimum oligosaccharide sequence within heparin that binds annexin V was identified. Affinity chromatography studies confirmed the  $\text{Ca}^{+2}$  dependence of this binding interaction. Analysis of the data obtained from surface plasmon resonance afforded a  $K_d$  of  $\sim 21$  nM for the interaction of annexin V with end-chain immobilized heparin and a  $K_d$  of  $\sim 49$  nM for the interaction with end-chain immobilized heparan sulfate. Isothermal titration calorimetry showed the minimum annexin V binding oligosaccharide sequence within heparin corresponds to an octasaccharide sequence. The  $K_d$  of a heparin octasaccharide binding to annexin V was  $\sim 1$   $\mu\text{M}$  with a binding stoichiometry of 1:1.

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**Key words:** Heparin; Surface plasmon resonance; Annexin V; Octasaccharide; Isothermal titration calorimetry

## 1. Introduction

Annexins are a family of homologous proteins that are widely distributed and ubiquitous in eukaryotes [1–5]. A distinctive feature of annexins is their calcium-dependent binding to the surface of phospholipid membranes. The *in vivo* functions of these proteins are unclear, but their close associations with cell membranes suggest their involvement in various processes, including: membrane trafficking (e.g. exocytosis, endocytosis), cytoskeletal-membrane interactions, signal transduction, cell adhesion, and regulation of the activities of ion channels and other membrane-bound proteins. Though annexins were originally considered intracellular proteins, many extracellular events are now proposed to be annexin-mediated.

Annexins II and V have been studied most extensively in terms of extracellular function. Annexin II acts as a co-receptor for the plasminogen/tissue plasminogen activator [6], and as a receptor for the human cytomegalovirus [7] and the matrix protein, tenascin-C [8]. Annexin V, known in this context as anchorin II, interacts strongly with collagen types II and X [9]. A role in viral entry and infection by influenza [10] and hepatitis B [11] viruses also has been demonstrated for annexin V. Several annexins, particularly annexin V, exhibit potent anticoagulant activity *in vitro*.

Proteoglycans (i.e. macromolecules consisting of a protein

core and glycosaminoglycan side chains), which are found in the extracellular matrix, on the cell surface and in secretory granules are involved in a wide range of activities [12]. Some of their functions are likely to depend on the direct interactions between glycosaminoglycans (GAGs) and other molecules [13]. Calcium-dependent lectin activity [14] and/or binding to specific sialoglycoproteins and GAGs has been identified in several annexins. The heparin binding properties of annexin II tetramer have been characterized and a potential heparin-binding site identified [15,16]. The binding of annexins IV, V, and VI to GAGs, including heparin, heparan sulfate, or chondroitin sulfate, has been reported recently [17]. These studies have shown that the annexins express different GAG preferences and that GAG binding may be calcium dependent or independent. The present studies use affinity chromatography, isothermal titration calorimetry (ITC), and surface plasmon resonance (SPR) to probe the energetics and kinetics of the annexin V-heparin interaction. These studies also enable the determination of the minimum oligosaccharide sequence within heparin that binds to annexin V. Identification of a unique carbohydrate-binding site for annexin V will provide a basis for understanding the mechanism of the interaction.

## 2. Materials and methods

Recombinant rat annexin V was prepared as described previously [18]. Heparin, crude containing peptidoglycan ( $M_r$  avg = 15 500; 150 U/mg), pharmaceutical (USP lyophilized,  $M_r$  avg = 14 000; 177 U/mg) and low molecular mass (LMW) ( $M_r$  avg = 7000; 74 U/mg) from porcine intestinal mucosa, sodium salts, were prepared by Celsus Laboratories (Cincinnati, OH, USA). Peptidoglycan heparan sulfate ( $M_r$  avg = 11 000) was prepared from bovine kidney tissue in our laboratory [19] by the method of Lyon and Gallagher [20]. A heparin-derived octasaccharide was prepared by heparin lyase I (EC 4.2.2.7) treatment of pharmaceutical heparin and purified to homogeneity by gel permeation and strong anion exchange chromatography as previously described [21]. Biotin-x-hydrazide was from Calbiochem (La Jolla, CA, USA), and biotin-LC-hydrazide, succinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin) and 1-ethyl-3-(dimethyl-aminopropyl) carbodiimide hydrochloride (EDC) were from Pierce, (Rockford, IL, USA). Guanidinium chloride, piperazine HCl, *N*-ethylmaleimide, phenylmethylsulfonyl fluoride, trichloroacetic acid, deuterium oxide (99.96 atom %) and chondroitinase ABC were obtained from Sigma (St. Louis, MO, USA). Heparin lyases were from Siekagaku America, (Rockville, MD, USA) and heparin disaccharide standards were from Grampian Enzymes (Aberdeen, UK). Ultrapure urea was from Life Technologies (Grand Island, NY, USA). Triton X-100, EDTA and polyethylene glycol were from Fisher Scientific (Fair Lawn, NJ, USA). Proteinase K was from Boehringer Mannheim (Germany). DEAE-Sephacel matrix was from Pharmacia Biotech (Uppsala, Sweden). All other reagents were analytical grade. Spectrophotometric measurements were carried out on a Shimadzu model UV-2101PC UV-vis spectrophotometer. ITC was performed on a Calorimetry Sciences Corporation Model 4200 (Provo, UT, USA). SPR studies were

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done on a BIAcore 2000 instrument at the Biopolymer Facility of Ohio State University (Columbus, OH, USA).

### 2.1. Affinity chromatography

A heparin-Sepharose column was equilibrated with annexin buffer (50 mM HEPES at pH 7.4, containing 0.1 mM DTT). Annexin V (1.5 mg in 1 ml of annexin buffer) was loaded on the column and the column was then washed with annexin buffer. The column was eluted using a salt gradient (0.1 M–1.0 M) in the same buffer and 1 ml fractions were collected. Protein was detected at 280 nm and salt concentrations were determined by measuring the conductivity. This experiment (loading, washing and salt gradient elution) was repeated using an annexin buffer containing 5 mM CaCl<sub>2</sub>.

### 2.2. Isothermal titration calorimetry

Annexin V (50 μM, 1 ml) was prepared in 50 mM HEPES at pH 7.4, containing 0.1 mM DTT and 5 mM CaCl<sub>2</sub>. LMWH samples were prepared in the same buffer at concentrations ranging from 600 to 155 μM. The sample cell was loaded with 1 ml of the protein sample and the system was equilibrated for 15 min. LMW heparin was titrated into the cell using a 100 μl syringe (10 injections, 10 μl each). Experiments were performed at 25°C. Calorimetry data were analyzed using Origin 2.88 software. Experimental details and data processing relied on previously described methods [22].

### 2.3. Immobilization of glycosaminoglycans

Peptidoglycan heparin and heparan sulfate were biotinylated in the core peptide using NHS-LC-biotin to afford reducing-end biotinylated heparin and heparan sulfate. Pharmaceutical heparin was periodate oxidized at residues within the polysaccharide chain and reacted with biotin-hydrazide to afford mid-chain biotinylated heparin [23]. A streptavidin sensor surface (SA sensor chip, Pharmacia AB) was pre-treated with three 5 μl injections of 50 mM NaOH in 1 M NaCl, to remove any non-specifically bound contaminants. A 5 μl injection of reducing-end biotinylated heparin in HEPES running buffer (HBS) (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, pH 7.4 containing 0.005% (v/v) P-20 surfactant) was made followed by a 10 μl injection of 2 M NaCl. The other three flow cells of the sensor chip were similarly treated to immobilize reducing-end biotinylated heparan sulfate, mid-chain biotinylated heparin and buffer only to serve as a control. An approximately 100 RU increase was detected in each of the three glycosaminoglycan containing flow cells when compared to the control cell.

### 2.4. Kinetic measurement of annexin-glycosaminoglycan interactions

Typically, a 15 μl injection of annexin (at a concentration range of 0.25–1.5 μM in annexin buffer containing 5 mM CaCl<sub>2</sub>) was made at a flow rate of 5 μl/min. At the end of the sample plug, the same buffer (without added annexin) was flowed past sensor the surface to allow dissociation. After a suitable dissociation phase the sensor surface was regenerated for the next annexin sample using a 10 μl pulse of regenerating buffer (50 mM HEPES, pH 7.4 containing 0.1 mM DTT and 0.5 M NaCl). The same solution was flowed past all four sensor surfaces in sequence and the response was monitored as a function of time (sensorgram) at 25°C. The control cell was used to subtract the contribution of non-specific interaction with the dextran matrix itself. Kinetic parameters were evaluated using the BIA Evaluation software according to the manufacturer's methods by first evaluating the dissociation rate constant  $k_d$  using  $dR/dt = -k_d R$  where  $R$  represents the sensor response. The association rate constant was determined from the slope of a plot of the observed association rate constant  $k_s$  as a function of ligand concentration, according to  $k_s = k_d + k_a C$  where  $k_a$  is the association rate constant and  $C$  represents the ligand concentration. The dissociation constant,  $K_d$ , was obtained from the ratio of  $k_d/k_a$ .

### 2.5. NMR analysis

For <sup>1</sup>H-NMR spectroscopy, approximately 1 mg of the heparin octasaccharide sample was exchanged three times with 1 ml portions of <sup>2</sup>H<sub>2</sub>O and lyophilized. The thoroughly dried sample was redissolved in 0.7 ml <sup>2</sup>H<sub>2</sub>O, and spectra were obtained using a UNITY-500 spectrometer at the operating frequency of 500 MHz equipped with a VXR 5000 computer system from Varian Instruments. Probe temperature was 298 K.

## 3. Results

### 3.1. Affinity chromatography

Annexin V was loaded on the heparin-Sepharose column in the absence and presence of calcium chloride. In the absence of CaCl<sub>2</sub> little if any protein bound to the column and most protein was recovered in the wash. In the presence of CaCl<sub>2</sub>, the protein bound to the column. Bound protein was eluted from the column as soon as the salt gradient was started, i.e. ~0.1 M NaCl. This experiment confirmed the calcium-dependence of the annexin V-heparin interaction. The concentration of sodium chloride required to elute heparin-binding proteins from heparin-Sepharose by disrupting ion-pairing interaction is often used to estimate binding avidity [24,25]. However, in the present case, calcium binding and concomitant heparin binding to the protein cannot be differentiated. Potentially, either binding interaction can be antagonized by increased ionic strength. Thus, an alternative method was necessary to study heparin binding to annexin V.

### 3.2. Isothermal titration calorimetry

ITC is useful for measuring the energetics of heparin-protein binding [22] and thus, was applied to understand the interaction of annexin V with heparin. The heat of interaction measured by ITC can be used to calculate the  $K_d$  and  $n$  (the number of ligand interactions per mol of macromolecule). The titration between 50 μM annexin V and 156 μM LMW heparin gave fitted heats of interaction of  $\Delta H$  of  $-8.24$  kcal/mol yielding a  $K_d$  of 1.7 μM, and an  $n$  of approximately 3.7–4 mol of annexin V per mol of LMW heparin (at 25°C in annexin buffer containing 5 mM CaCl<sub>2</sub> at pH 7.4) (Fig. 1). Based on the  $M_{r,avg}$  of 7000 determined for LMW heparin it has ~20–24 saccharide residues per GAG chain. Thus, the calculated  $n$  value corresponds to a heparin-binding site for annexin V consisting of 6–8 saccharide residues. To test this result, an octasaccharide was prepared from heparin by partial depolymerization with heparin lyase I and purified to homogeneity by gel permeation chromatography and strong anion exchange (SAX)-HPLC. Capillary and gel electrophoresis confirmed the purity of the octasaccharide to be >95% [21]. The

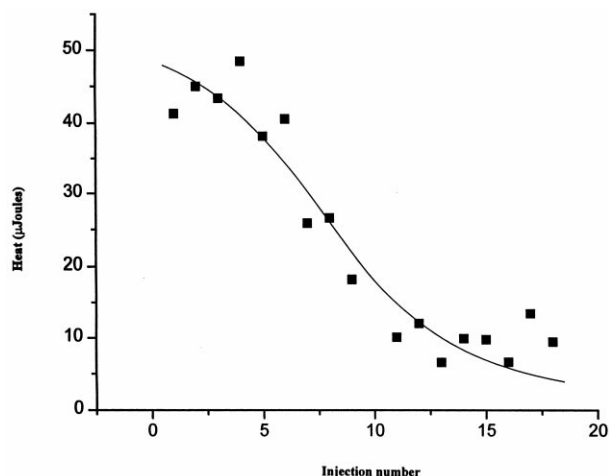


Fig. 1. Binding isotherm of the interaction between annexin V and LMW heparin. 50 μM of annexin V was titrated against 156 μM of LMW heparin at 25°C in an annexin buffer containing 5 mM CaCl<sub>2</sub> at pH = 7.4.

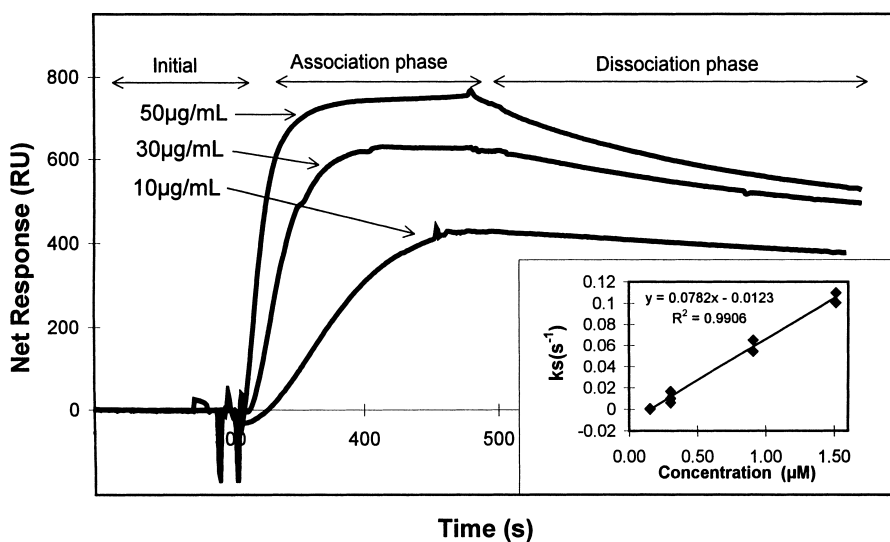


Fig. 2. SPR sensograms of annexin V-heparin (reducing-end biotinylated) interactions. Varying concentrations of annexin V (50, 30 and 10  $\mu\text{g/ml}$ ) were allowed to flow over a BIAcore chip with reducing-end biotinylated heparin immobilized on the surface.

primary structure of this octasaccharide was assigned using 1D  $^1\text{H-NMR}$  (Fig. 3). Isothermal titration calorimetry experiments with the octasaccharide and annexin V afforded a  $K_d$  of interaction of  $\sim 1 \mu\text{M}$  and a binding stoichiometry of 1:1.

### 3.3. Kinetic measurement of annexin-glycosaminoglycan interactions

Sensorgrams for the binding of annexin to the reducing-end immobilized heparin are shown in Fig. 2. The initial part of these curves represents a buffer flowing past the sensor surface. The second and rising part of each curve corresponds to the response of the sensor surface observed as a sample injection volume flows past the immobilized GAGs. The final portion of the curves corresponds to the dissociation of bound protein after the sample volume has finished and the buffer flows past the sensor surface again. The dissociation rate constant,  $k_d$ , was evaluated from each trace and used to calculate the observed association rate constant ( $k_s$ ). A plot of  $k_s$  as a function of ligand concentration yields a slope of the association rate constant,  $k_a$ . The ratio  $k_a/k_d$  generates the overall association constant or dissociation constant. The binding curves are relatively steep in the association phase (Fig. 2), which is indicative of the high affinity of annexin V for heparin and a quick association step. The dissociation curves are very gradual and shallow, which indicates that it is a tight interaction. Kinetic analysis of the interaction between annexin and reducing-end immobilized heparin afforded a  $K_a$  value of  $4.9 \times 10^7 \text{ M}^{-1}$  ( $K_d$  of 21 nM). Identical calculations for mid-chain immobilized heparin and reducing-end immobilized heparan sulfate yielded  $K_a$  ( $K_d$ ) values of  $2.0 \times 10^7 \text{ M}^{-1}$  ( $K_d$  of 49 nM) and  $2.4 \times 10^7 \text{ M}^{-1}$  ( $K_d$  of 41 nM), respectively.

## 4. Discussion

The present studies afford data on the energetics and kinetics of the calcium-dependent annexin V-heparin interaction. Several lines of evidence indicate that the interaction is not simply based on the affinity of annexins for polyanions, although the sulfate moieties are clearly important for binding [17]. The specificity of the annexin V-heparin interaction is

demonstrated by the weak interactions of sucrose octasulfate, as compared to a heparin-derived octasaccharide. A similar conclusion is drawn from the observation that annexin V binds heparin and heparan sulfate but not chondroitin sulfate [17].

Waisman and coworkers have suggested, based on their studies of annexin II, that heparin binds at a site that conforms to a consensus heparin binding sequence in domain 4 [15]. A similar binding site, consisting of Arg-283, Lys-284, Arg-287, and Lys-288, can be predicted from the sequence of annexin V. In terms of molecular structure, this region of the protein is spatially distant from the annexin-membrane interfacial surface, which contains phospholipid and calcium binding sites. Instead, the consensus heparin binding sequences are located on the opposite protein surface, which faces the cytosol or extracellular milieu. Inspection of the rat annexin V crystal structure [26,27] suggests the presence of a second heparin binding sequence, Arg-205, Arg-206, and Lys-210 in domain 3. The two putative heparin sites thus contain sym-

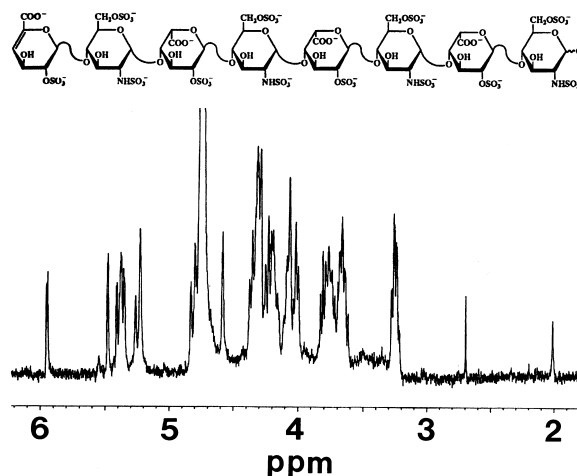


Fig. 3. The 1D  $^1\text{H-NMR}$  of the octasaccharide identified as the most probable annexin V binding oligosaccharide. The structure as deduced by the NMR is also shown.

metrical clusters of basic amino acids spaced 2–3 amino acids apart, a pattern that is predicted to recognize heparin or highly sulfated regions of heparan sulfate [13]. In annexin V, the putative domain 4 site exhibits a classical B-B-X-X-B-B pattern, while the domain 3 site lacks the fourth basic residue. In the tertiary structure, each site presents the basic residues on the same helical face (helices 3C or 4C, respectively). Molecular modeling using a 12-saccharide heparin molecule in a helical conformation ([28]; Protein Data Bank deposition number, 1HPN) suggests that an octasaccharide is the smallest unit that would span the two predicted sites in annexin V without significant rearrangement of the protein structure. Thus the model agrees well with the experimental data presented herein. Further studies on the interaction of annexin V with heparin and other oligosaccharides are in progress and should help elucidate the structural requirements for binding and the mechanism of this interaction.

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